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Biological Activity of Naphthoquinones

Cytotoxic activity against eukaryotic plant cells and against eukaryotic human cells
Antimicrobial activity of Naphthoquinones against selected microorganisms

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Mestrado Integrado em Ciências Farmacêuticas

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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

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I - Resumo

A resistência antimicrobiana ocorre quando os microrganismos (fungos, bactérias, vírus e parasitas) adquirem a capacidade de alterar a sua resposta aos fármacos antimicrobianos. Esta capacidade é adquirida através de alterações genéticas, em resultado de um processo de evolução natural. No entanto esse processo encontra-se de tal forma acelerado que as infeções provocadas por bactérias resistentes aos agentes antimicrobianos são uma realidade cada vez mais frequente. De facto, a resistência aos antimicrobianos é já um problema de saúde pública a nível mundial que tem como consequências o aparecimento de infeções persistentes, o aumento dos custos de internamento e a incapacidade e morte dos indivíduos que adquirem estas infeções. Estima-se que, atualmente, cerca de 700.000 pessoas morram por ano em todo o mundo, vítimas das bactérias multirresistentes, para as quais não há alternativas terapêuticas. Prevê-se que em 2050 estas infeções sejam mais mortíferas que o cancro, se nada for feito para as combater.

As causas na origem deste problema passam pela utilização excessiva dos antibióticos, pela prescrição de antibióticos que não são os mais adequados para as bactérias em questão, pelo acesso facilitado a antibióticos em países onde os estes estão disponíveis como medicamentos não sujeitos a recita médica, pelo uso excessivo na criação de animais, pela utilização como pesticida em árvores de fruto e pelo parco investimento que tem havido no campo do desenvolvimento de novos antibióticos.

Este tópico é frequentemente alvo de debate por parte da Organização Mundial de Saúde e ao longo dos últimos anos têm sido estudadas várias formas de fazer frente a este problema: apostar na prevenção da transmissão das bactérias resistentes e no uso responsável dos antibióticos, bem como investir na investigação de novos fármacos capazes de suprimir o crescimento destas bactérias. A realidade é que existem muito poucas moléculas em desenvolvimento e apenas uma pequena porção destas poderão ser consideradas como tratamentos inovadores. As restantes não são mais do que modificações das moléculas já existentes, o que significa que as bactérias podem ser naturalmente resistentes a estes novos antibióticos por atuarem com mecanismos semelhantes aos dos antibióticos aos quais as bactérias já adquiriram resistência.

A Organização Mundial e Saúde criou uma lista com os microrganismos que são considerados mais preocupantes, no que diz respeito à rapidez com que estão a ser esgotadas as alternativas disponíveis para os combater. O objetivo de tal lista é o de orientar a investigação e desenvolvimento de novas moléculas direcionadas para esses microrganismos. As bactérias gram-negativas estão no topo da lista, uma vez que têm

desenvolvido novas resistências a um ritmo alucinante, tendo inclusive adquirido resistência a antibióticos de última linha, como os *carbapenems*. Estas bactérias são as mais difíceis de lidar nos cuidados de saúde, estando muitas vezes relacionadas com infeções nosocomiais.

A investigação de novas alternativas terapêuticas está a ser conduzida por duas vertentes. Por um lado, estão a ser testados produtos que não costumam ser usados para combater as bactérias, mas que podem ser usados em combinação com os antibióticos para aumentar a resposta antimicrobiana, ou até serem usados na prevenção da infeção. São exemplos desses produtos as vacinas, os anticorpos e os probióticos. Por outro lado, estão a ser analisadas as bases de moléculas já existentes, para encontrar as que revelem atividade antimicrobiana, com especial foco para os produtos de origem natural ou compostos sintéticos derivados dos mesmos. As fontes naturais dos novos antibióticos incluem plantas, fungos, líquenes, endófitos, plantas marinhas e corais. Dentro deste grupo de compostos naturais, os compostos fenólicos destacam-se pelas capacidades de defesa que conferem às plantas. Estes compostos já mostraram possuir propriedades antimicrobianas e têm potencial para serem usados em humanos. Um dos mecanismos pelos quais parecem exercer a sua atividade antimicrobiana é através da inativação das enzimas celulares.

As naftoquinonas são compostos fenólicos derivados do naftaleno. São pigmentos bastante conhecidos e estão amplamente distribuídas na natureza, sendo encontradas maioritariamente em plantas, mas também em fungos e em alguns animais. Estes compostos têm sido amplamente estudados, nomeadamente pelas suas propriedades anticancerígenas. No entanto exibem também atividade antibacteriana, antifúngica, anti-inflamatória, antiviral, antimalárica, entre outras. O mecanismo de ação das naftoquinonas envolve frequentemente a formação de espécies reativas de oxigénio, através de reações de oxidação/redução. Outros mecanismos propostos para a sua atividade biológica são reações ácido-base, alquilação e indução de quebra do ácido desoxirribonucleico e inativação de proteínas. As naftoquinonas são compostos altamente reativos com a capacidade de aceitar um ou dois eletrões, formando radicais aniónicos. A sua atividade varia consoante os substituintes presentes e a sua posição no anel da naftoquinona.

Neste projeto de investigação foram testados oito compostos pertencentes à classe das naftoquinonas, com o objetivo de conhecer o seu potencial antimicrobiano, determinando a sua concentração inibitória mínima. Dois dos compostos testados têm origem natural, Lawsone (2-hidroxi-1,4-naftoquinona e Juglone (5-hidroxi-1,4-naftoquinona), e os restantes são derivados sintéticos dos primeiros: 2-metoxi-1,4-naftoquinona, 1,2-naftoquinona, 2,3-

dicloro-1,4-naftoquinona, 2,3-dibromo-1,4-naftoquinona, Menadione (2-metil-1,4-naftoquinona), 2-bromo-1,4-naftoquinona.

O foco deste projeto foi a otimização das metodologias e a seleção das condições experimentais ideias para testar o potencial antimicrobiano de várias naftoquinonas. Uma vez que a amostragem é reduzida, as experiências realizadas carecem de significância estatística, um problema inerente à duração do estágio e ao tipo de metodologias utilizado. Seria necessário mais tempo para repetir cada uma das experiências, de modo a permitir uma análise estatística fiável dos resultados.

Foi testada inicialmente a gama de atividade dos oito compostos, utilizando células de *Daucus carota.* Em seguida foram selecionadas as concentrações de naftoquinonas a utilizar nas fases posteriores do projeto, nomeadamente nos testes de toxicidade em células humanas. Estes testes pretendiam calcular a concentração de cada naftoquinona necessária para inibir 50% do crescimento das células. Foi possível concluir que, à exceção do composto 2-hidroxi-1,4-naftoquinona, todos os compostos testados, dependendo da concentração utilizada, poderão ser citotóxicos para as células humanas, sendo que a 2-metoxi-1,4-naftoquinona se encontra bastante próxima do limiar entre a toxicidade e a toxicidade moderada.

A sensibilidade às naftoquinonas foi testada recorrendo a diferentes espécies de microrganismos: *Micrococcus luteus*, bactéria *gram*-positiva, *Escherichia coli*, bactéria *gram*-negativa comensal no organismo humano e *Saccharomyces cerevisiae*, levedura. Através da análise da concentração inibitória mínima (que corresponde a uma redução de 80% no crescimento das colónias), observou-se que o composto 2-hydroxy-1,4-naphthoquinone revelou atividade antimicrobiana para os três microrganismos testados, mostrando um largo espectro de ação, e o composto 2-methoxy-1,4-naphthoquinone mostrou capacidade para inibir o crescimento da *Escherichia coli* e da *Saccharomyces cerevisiae*, mas não do *Micrococcus luteus.*

Os referidos compostos revelaram uma atividade antibacteriana bastante satisfatória, sendo fortes candidatos para vir a combater infeções causadas por bactérias multirresistentes, como é o caso de algumas estirpes de *Escherichia coli.* Mais testes são necessários para validar estatisticamente estas conclusões e para concluir sobre a capacidade destas moléculas inibirem o crescimento das estirpes multirresistentes.

Palavras-chave: Naftoquinonas, Atividade antimicrobiana, Resistência aos antimicrobianos, Atividade biológica.

II - Abstract

Infections caused by bacteria resistant to antimicrobial therapy are more and more frequent. Bacteria acquire resistance through genetic mutations that occur naturally, but this process has been accelerated due to the misuse of antibiotics. In fact, antibiotics have been overprescribed and even used over the counter, not to mention the extensive use of antibiotics in food-producing animals and plants. Antimicrobial resistance has become a worldwide public health problem, causing persistent infections, increased treatment costs, disability and death. Bacteria are growing resistant to last resource treatments and the pipeline for antimicrobial drugs is practically empty. It is urgent to invest in Research and Development to find new alternatives to fight multi-resistant bacteria, specifically for Gram-negative bacteria, which raise more concern because of the threat they represent in health care.

Natural products constitute a possible solution for the antimicrobial resistance problem, due to their antimicrobial and resistance-modifying proprieties. Natural phenolic compounds can be emphasised for their potential to be used in humans as antibiotics.

Naphthoquinones are phenolic compounds widespread in nature, that have been profoundly studied for their antitumoral proprieties, and also exhibit a strong antibacterial activity. Their mechanism of action often comprises the formation of reactive oxygen species, through oxide/reduction reactions, and they can accept one or two electrons, forming highly reactive radicals.

Eight naphthoquinones have been tested for their toxicity and then used to test the sensibility with *Micrococcus luteus, Escherichia coli* and *Saccharomyces cerevisiae.*

Every compound tested was toxic on human cells, except for 2-hydroxy-1,4-naphthoquinone. The compound 2-methoxy-1,4-naphthoquinone was in the borderline between toxicity and moderate toxicity. The first compound revealed a broad range of activity, being able to reduce the growth of all the selected microorganisms. The second one only showed antimicrobial activity against *Escherichia coli* and *Saccharomyces cerevisiae.*

For statistically significative conclusions, experiments should be repeated, once this work was focused on optimizing the approaches and selecting the best experimental conditions.

Keywords: Naphthoquinones, Antimicrobial activity, Antimicrobial resistance, Biological activity.

III – Acknowledgments

Firstly, I would like to acknowledge the European Commission, the University of Lisbon and the University of Veterinary and Pharmaceutical Sciences Brno, for allowing me to live the Erasmus+ experience, which was extremely enriching both in the academic field and in the cultural experience. I will always look back to this period of my life with nostalgia.

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Last, but not least, I would like to thank my family and my friends for their patience, support and encouragement. I want to apologise them for being away for so long.

I would like to leave a special thank you to the most fundamental pieces of my whole existence, my mum and dad, for everything I could never put into words.

Lisbon, November 2017

Inês Pedrosa

IV - Acronyms

51: Juglone (5-hydroxy-1,4-naphthoquinone)

482: 2-methoxy-1,4-naphthoquinone

552: 1,2-naphthoquinone

553: 2,3-dichloro-1,4-naphthoquinone

554: Lawsone (2-hydroxy-1,4-naphthoquinone)

561: 2,3-dibromo-1,4-naphthoquinone

562: menadione (2-methyl-1,4-naphthoquinone)

563: 2-bromo-1,4-naphthoquinone

CSF: Cerebral Spinal Fluid

DCF: Dichlorofluorescein

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

E. coli: *Escherichia coli*

ESBL: Extended-spectrum Beta-lactamases

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FBS: Fetal Bovine Serum

H2DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate

IC50: Inhibitory concentration at half maximum

KH2PO4: Potassium Dihydrogen Phosphate

MF: McFarland (unit of density of suspensions)

MIC: Minimum Inhibitory Concentration

M. luteus: *Micrococcus luteus*

NADH: Nicotinamide Adenine Dinucleotide

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

PBS: Phosphate Buffered Saline

S. cerevisiae: *Saccharomyces cerevisiae*

R&D: Research & Development

ROS: Reactive Oxygen Species

RPMI: Roswell Park Memorial Institute medium

THP-1: Tamm-Horsfall Protein 1 cell line

TMP/SMX: Trimethoprim / Sulfamethoxazole

WHO: World Health Organization

WST: Tetrazolium salt

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1. Introduction

1.1 Antimicrobial Resistance

Antimicrobial resistance occurs when microorganisms (fungi, viruses, bacteria and parasites) are able to change their response to antimicrobial drugs, through genetic changes (1). The development of resistant strains occurs naturally, through several pathways, depending on the target of the drug: modification of the target, prevention of cell penetration, expulsion via efflux pumps or degradation/modification (inactivating proteins) of the drug (2). However, this process is being facilitated and accelerated by the misuse of those medicines in both people and animals. The outcome is ineffectiveness of standard treatments, followed by the spreading of resistant microorganisms and resulting in persistent infections, disability and death (1). It is estimated that infection by multi-resistant bacteria already kills 700.000 people in the whole world every year, and it is predicted that this number will grow until the point where those infections will kill more people than cancer does (3).

It is well known that antimicrobial resistance is a worldwide problem that threatens the core of modern medicine and the effectiveness of a global public health response to treat infectious diseases (4). In fact, the World Economic Forum has identified antimicrobial resistance as a "global risk beyond the capacity of any organization or nation to manage or mitigate alone"(5), once "the indirect impact of antimicrobial resistance extends beyond increased health risks", particularly in the economic field, as it reduces productivity and increases the costs of treatment (4). The causes that led to this global Public Health problem are diverse. One of the most relevant is the overuse of antibiotics, which is deeply related to inappropriate prescription of antibiotics and the facilitated access to them, once "in many countries, antibiotics are unregulated and available over the counter without a prescription." Other causes are: extensive agricultural use, to prevent infection and promote growth of animals and to act as pesticide on fruit trees, low availability of new antibiotics and regulatory barriers (6).

As a matter of fact, one of the problems with antimicrobial resistance is that the pipeline for new antibiotics is practically empty (7). This is happening because, for pharmaceutical industries, the antibiotic development ceased to be an economically desirable investment (6). As Dr Tedros Adhanom Ghebreyesus, director-general of the World Health Organization (WHO), said: "There is an urgent need for more investment in research and development for antibiotic-resistant infections, otherwise we will be forced back to a time

when people feared common infections and risked their lives from minor surgery." From the 51 molecules that are currently in development, that may or may not be able to treat multi-resistant bacteria, only eight have been classified by WHO as "innovative treatments that will add value to the current antibiotic treatment arsenal." The remaining molecules are simply modifications of already existing medicines, which means that the bacteria might be able to resist to the new drugs, based on the similarity of the mechanism of action (7).

This work falls into the second strategic objective from WHO Global Action Plan on Antimicrobial Resistance: "Strengthen the knowledge and evidence base through surveillance and research", namely the research for the development of new treatments (4). Nevertheless, other approaches, such as prevention of infections and rational use of existing and future antibiotics, play a crucial role in fighting antimicrobial resistance (8).

In February 2017, WHO published a list with the "priority pathogens" as a tool to promote and guide Research & Development (R&D) in what comes to new antibiotics. This list points out the microorganisms of more concern, namely gram-negative bacteria resistant to multiple antibiotics. *Escherichia coli (E. coli),* one of the bacteria used in this experiment, is among this multi-resistant bacteria group classified as critical (the most urgent level from the three presented in the WHO list). This urgent character is due to the threat those bacteria represent in nursing homes, hospitals and among patients with ventilators or catheters, once those bacteria already become resistant to last resource treatments for multi-resistant bacteria, such as third generation cephalosporins and carbapenems (8). "Without effective drugs, doctors cannot treat patients. Within a generation, without new antibiotics, deaths from drug resistant infection could reach ten million per year. Without new medicines to treat deadly infections, lifesaving treatments like chemotherapy and organ transplant, and routine operations like caesareans and hip replacements will be potentially fatal", noticed Tim Jinks, head of drug resistant infections at the Wellcome Trust (9).

1.2 Innovative approaches as antimicrobial drugs

Between the years 1962 and 2000, pharmaceutical companies were focused not in the development of new classes of antibiotics, but in the development of different analogues of the antibiotics that already existed. This might have been due to the smaller potential of toxicity of the analogues when compared to new classes (10).

Currently, non-traditional products are being studied to combat bacteria. The products in development cover well-known medical interventions, as immunotherapies and vaccines, and completely new types of therapies as well. Those products won't replace antibiotic

therapy, but they can be combined to obtain better results or even used for prevention (11). The Pew Charitable Trusts developed a list (that is continuously being updated) with the "Non-traditional Products for Bacterial Infections in Clinical Development". This list was updated the last time on March 2017, containing 32 new "products with the potential to treat or prevent infections caused by bacterial pathogens considered to be urgent, serious, or concerning threats" (12). There are four main classes of non-traditional products in development for the U.S. market, as showed in Table 1.1 (11).

Table 1.1: Non-traditional products in the pipeline

Product	Description
Vaccines	Agents that stimulate the immune system in order to identify and destroy pathogens, protecting the patient from getting infected.
Antibodies	Proteins naturally generated by the immune system to recognize and help destroy potentially harmful pathogens. New therapies are taking advantage of specific targeting capacities that antibodies have, allowing them to bind to bacteria, inactivating them in different ways.
Probiotic	Live microorganisms that help preserve and re-establish populations of helpful bacteria in the human gut. Administrating broad spectrum antibiotics frequently indiscriminately kills gut bacteria, which makes side effects and colonization by harmful bacteria more likely to occur. Administering probiotics alongside antibiotics helps to alleviate these risks.
Lysin	Derived from bacteriophages (viruses that infect bacteria) that target and break up bacterial cell wall architecture.

The biggest group of non-traditional products in investigations are vaccines, followed by antibodies (11).

At the same time, R&D on natural products with antibiotic activity, can be a key-point to solve the problem of antimicrobial resistance. "Natural sources of new antimicrobials and resistance-modifying agents include land plants, fungi, lichens, endophytes, as well as marine plants, seaweeds, corals, and other marine microorganisms", and molecules of different classes are continuously being found (13). From all the classes, we can highlight the phenolic compounds, with importance in plants for defence against microorganisms (14), and that have shown antibacterial activity, with potential for human use, either alone or in combination with other antibiotics (15). The research on natural products is based on the re-evaluation of know natural products (either biological or from synthesis), by screening for activity against each bacteria (13).

Phenolic compounds act as antimicrobial agents, by inactivating cellular enzymes. The intensity of their activity depends on the rate of penetration through the cell membrane. The antimicrobial potential of these compounds vary with the number and the position of substitutions in the benzene ring (16).

1.3 Naphthoquinones and their potential

Naphthoquinones are phenolic compounds (17). They are a type of pigments widespread in nature, from plants and fungi until some animals (18). These compounds exhibit strong action as antibacterial, antifungal, antiviral, antimalarial and antitumoral agents (19), and other interesting activities, such as anti-inflammatory, antiplatelet, antiallergic and antithrombotic (20). The biological activity of naphthoquinones is related to their acid/base and oxide/reduction properties, both of which can be modulated (19), being highly reactive compounds (21), and, as so, often comprises the formation of reactive oxygen species (ROS) (19). Other mechanisms have been proposed, such as intercalation in deoxyribonucleic acid (DNA), alkylation of DNA or the inhibition of particular proteins (18).

Chemically, naphthoquinones derive from the naphthalene and can present two carbonyl groups in the 1,2-positions (1,2-naphthoquinones) or 1,4-positions (1,4-naphthoquinones) (21) (Figure 1.1), being able to form anionic radicals by accepting one or two electrons (20) (Figure 1.2). The activity of the naphthoquinones varies depending on the substituents and their position in the naphthoquinone ring. Scientists have studied the antibacterial and antifungal activities with non-standardized methods and it can be considered that the methods based on “broth dilution antibacterial and antifungal susceptibility testing of aerobic bacteria and yeasts” are among the best (18).

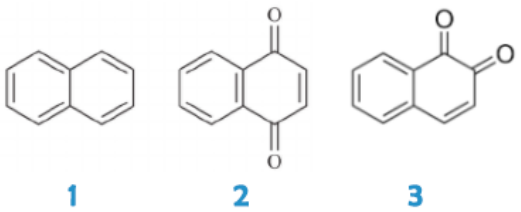

Figure 1.1: Chemical structure of naphthalene (1), 1,4-naphthoquinone (2) and 1,2-naphthoquinone (3) (Adapted from ChemSpider)

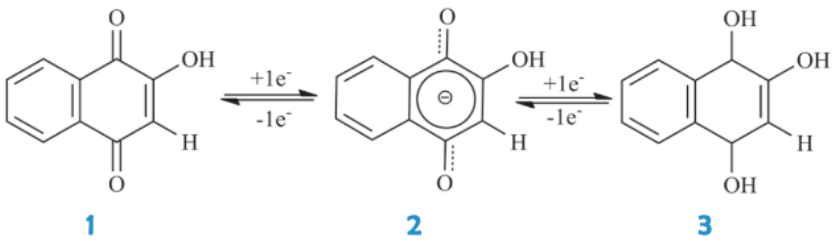

Figure 1.2: Redox properties of quinone (1), forming semiquinone (2) and hydroquinone (3) (19)

From all the Naphthoquinones, the derivates of 1,4-Naphthoquinone have a bigger interest for science due to their strong biological activity (18). From this family of quinones, juglone, plumbagin and lawsone are the most widely spread ones, and so, the most studied as well (22).

Juglone, or 5-hydroxy-1,4-naphthoquinone (Figure 1.1 - 51), can be found in black walnut (Juglans nigra L.) (23). Plumbagin is present in the roots of the plant Plumbago zeylanica L (24) and has already been proved to have an excellent antimicrobial activity for S. aureus and C. albicans infections (25). Lawsone (Figure 1.1 – 554) is an orange dye found in the leaves of Lawsonia inermis (also known as henna plant) (26).

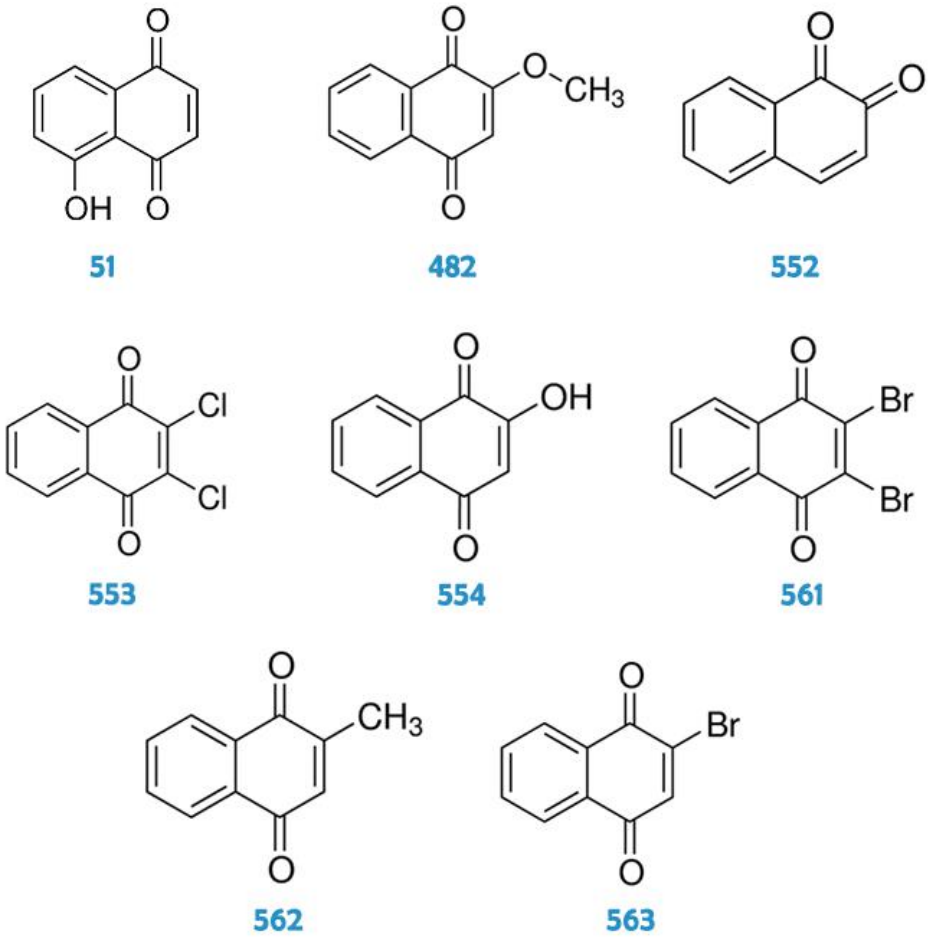

Figure 1.3: Chemical structure of the eight naphthoquinones used in this project **(51)** Juglone (5-hydroxy-1,4-naphthoquinone); **(482)** 2-methoxy-1,4-naphthoquinone; **(552)** 1,2-naphthoquinone; **(553)** 2,3-dichloro-1,4-naphthoquinone; **(554)** Lawsone (2-hydroxy-1,4-naphthoquinone); **(561)** 2,3-dibromo-1,4-naphthoquinone; **(562)** Menadione (2-methyl-1,4-naphthoquinone); **(563)** 2-bromo-1,4-naphthoquinone. **(Adapted from ChemSpider)**

1.4 - *Escherichia coli*, *Micrococcus luteus* and *Saccharomyces cerevisiae*

1.4.1 – *Escherichia coli*

Escherichia coli (E. *coli*) are Gram-negative bacteria, aerobic and anaerobic facultative, which means they are able to have a metabolism that is either respiratory or fermentative (27). E. *coli* are part of a bigger family of bacteria, the *Enterobacteriaceae* (28), which are commensal inhabitants of the gut of warm-blooded animals and humans. Most of E. *coli*'s strains are harmless (29) and can even be helpful to supress the growth of harmful bacteria and to synthetize appreciable amounts of vitamins (27). Nevertheless, some strains can cause severe diarrhoea, and when they invade sterile sites, all can cause infection. The most common site of infection is the urinary tract, but E. *coli* can also cause pelvic inflammatory disease, prostatitis, bacteraemia and hepatobiliary, peritoneal, pulmonary and cutaneous infections (30).

The treatment is done with antibiotics and guided by susceptibility tests. Most of the strains are resistant to tetracyclines and ampicillin, so other alternatives should be used, like piperacillin, cephalosporins, aminoglycosides, fluoroquinolones or trimethoprim/sulfamethoxazole (TMP/SMX). The increase of resistance factors in E. *coli* strains is notorious, and some multi-resistant strains can produce extended-spectrum beta-lactamases (ESBLs), causing them to be resistant to almost all the antibiotics available (broad-spectrum cephalosporins, penicillins and monobactams (30), and even carbapenems (31), which are last resource treatment). This is what makes E. *coli* one of the bacteria with top priority in the list of WHO that points out the microorganisms with higher priority to develop R&D for (8).

1.4.2 – *Micrococcus luteus*

Micrococcus luteus (M. *luteus*) are gram-positive cocci that have a positive catalase reaction (32) and a negative coagulase reaction (33). They are obligate aerobes and can be found in soil, dust, air and water. It is also part of the regular flora of the mammalian skin (34). While being in the human skin and eyes, M. *luteus* is harmless, but it can cause severe bacteraemia, exemplified by: "infection arising from a prosthetic heart valve, a ventriculovenous Cerebral Spinal Fluid (CSF) shunt to relieve hydrocephalus, or a polyethylene intravenous catheter" (33).

1.4.3 – *Saccharomyces cerevisiae*

Saccharomyces cerevisiae (S. *cerevisiae*) is a yeast and a very important model for understanding molecular and cellular processes in eukaryotes. This yeast is also used in industry, to make bread, wine , beer, enzymes, and pharmaceuticals (35). S. cerevisiae colonizes mucosal surfaces and it is part of the flora of the vagina and the gastrointestinal and respiratory tracts. Fungaemia is the most important condition caused by S. *cerevisiae*, not only in immunosuppressed patients, but also in healthy hosts. It can be caused by probiotic therapy with S. *cerevisiae* subtype *boulardii* or entry through central venous catheters. Therapy for fungaemia should contemplate the withdrawal of the probiotic treatment, administration of antifungal agents, and disposal of central venous catheters (36).

2. Project goals

The present research project was performed under the European Commission's Erasmus+ agreement, between University of Lisbon and University of Veterinary and Pharmaceutical Sciences Brno. The project main goal was to test the antimicrobial activity potential of new naphthoquinone compounds by evaluating their minimum inhibitory concentration (MIC).

To do so, the project was divided into three phases, each one with specific aim. The first phase was designed to find the range of activity of each of the eight tested compounds, using Daucus carota cells. The second phase intents to test the toxicity of the chosen compounds to human cells, searching for the least harmful concentration. In the last phase, the goal was to find the lowest MIC that is not toxic for the human cells.

All experiments were conducted between February and April 2017.

3. Materials and methods

3.1 - Materials

Eight different naphthoquinones, bought from Sigma Aldrich (Missouri, EUA), were selected to be tested in this project due to their potential for antimicrobial activity revealed in previous tests by the Czech investigation group (Table 3.1). Their chemical structure can be seen in the Figure 1.3.

Table 3.1: Identification of the Naphthoquinones

ID Number	Naphthoquinone
51	Juglone (5-hydroxy-1,4-naphthoquinone)
482	2-methoxy-1,4-naphthoquinone
552	1,2-naphthoquinone
553	2,3-dichloro-1,4-naphthoquinone
554	Lawsone (2-hydroxy-1,4-naphthoquinone)
561	2,3-dibromo-1,4-naphthoquinone
562	Menadione (2-methyl-1,4-naphthoquinone)
563	2-bromo-1,4-naphthoquinone

Chemicals:

Dimethyl Sulfoxide (DMSO) (Merck Millipore, Massachusetts, USA); Murashige-skoog medium (Duchefa Biochemie, Amsterdam, The Netherlands); Fluoresceine-diacetate (Sigma Aldrich, Missouri, USA); Tamm-Horsfall Protein 1 (THP-1) cells, purchased from the European Collection of Cell Cultures (Salisbury, UK); Phosphate Buffered Saline (PBS) (Merck Millipore, Massachusetts, USA); RPMI 1640 medium containing stabilized 2 mM L-glutamine (Biosera, France) supplemented with antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera)], and 10% Fetal bovine serum (FBS) (HyClone, UT, USA); RPMI 1640 serum free medium (Biosera, France); Tetrazolium salt (WST) (Sigma Aldrich, Missouri, USA); *Micrococcus luteus* (M. luteus) CCM 732, *Escherichia coli* CCM 7929 and *Saccharomyces cerevisiae* (S. cerevisiae) CCM 8191, currently in the cell bank from the laboratory.

3.2 - Cytotoxicity against eukaryotic plant cells

The Daucus carota's cells were suspended in sterile liquid medium (Murashige-skoog medium including vitamins - 4405,19mg/L, sucrose 30g/L, KH2PO4 0,2g/L, thiamin 0,9mg/L and 2,4 dichlorophenoxyacetic acid 0,2mg/L), gently mixed and filtered. Turbidity of cells'

suspension must be around 7 MF (McFarland units). We proceeded to the cultivation in 96-well microplate for determination of viability in the presence of naphthoquinones: the highest concentration of naphthoquinones used was 1280 µg/mL. Other concentrations were 640, 320, 160, 80, 40, 20, 10, 5 and 2,5 µg/mL, obtained by successive dilution on the microplate. We used 90µL of the Daucus carota's cells suspension and an initial 10 µL of the highest naphthoquinone solution in DMSO. The microplate was incubated for 24h and after incubation we added 10µg of Fluoresceine-diacetate for the dichlorofluorescein (DCF) assay. DCF assay is used to evaluate cellular oxidative stress (37), once naphthoquinones are known for causing oxidative stress to cells (38). "2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) is a non-fluorescent lipophilic ester that easily crosses the plasma membrane and passes into the cytosol, where it is rapidly cleaved by unspecific esterases. One of the reaction products is the non-fluorescent alcohol H_2DCF. The oxidation of this molecule to the fluorochrome DCF results in green fluorescence when excited with blue light. The brightness of this fluorescence is usually considered to reflect the extent to which reactive oxygen species (ROS) are present" (37). We measured the fluorescence using the microplate reader FLUOstar Omega (BMG Reader Labtech, Germany). The amount of fluorescence is proportional to the number of cells with intact cell membrane, and therefore proportional to the number of living cells.

3.3 - Cytotoxic activity against eukaryotic human cells

THP-1 human monocytic leukaemia cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 medium containing stabilized 2 mM l-glutamine (Biosera, France) supplemented with antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera)], and 10% FBS (HyClone, UT, USA). Cells were kept in an incubator at 37 °C in a water-saturated atmosphere of air containing 5% CO_2.

To determinate the viability of cells and their metabolic activity, we used the WST-1 test. WST is a tetrazolium salt that uses nicotinamide adenine dinucleotide (NADH) and Nicotinamide adenine dinucleotide phosphate (NADPH) as electron sources to act as an enzymatic dye. When WST is reduced, it changes colours and we can measure the amount of reduced WST-tetrazolium with an absorption measurement at 450 nm, which is proportional to the number of viable cells (39). Cells were counted in a Neubauer chamber in order to obtain a final concentration of 50 000 cells in each well. After being collected from the cell culture flask, cells were centrifugated and washed with PBS. Then serum free medium (RPMI 1640) was gently mixed with the cells and 100 µL were added into every well. Naphthoquinones were kept in stock solutions of 5 mg/mL, with DMSO, that was

diluted to achieve the concentration of 2,5 mg/mL, 1,25 mg/mL, 0,625 mg/mL and 0,3125 mg/mL. Each of the above solutions was diluted 20x in RPMI serum-free medium in order to achieve the final concentrations of: 5 µg/mL, 2,5 µg/mL, 1,25 µg/mL, 0,625 µg/mL and 0,3125 µg/mL in the assay microplate. Scheme of the dilution of the Naphthoquinones can be observed in the Figure 3.1. The microplate was then incubated at 37 °C in a water-saturated atmosphere of air containing 5% CO2 for 20h. After exposure to naphthoquinones, 10 µL of WST-1 were added into each well and incubated for 45 min. In wells where the cells were still viable, the colour switched from pink (colour of the medium) to yellow. Then the amount of reduced WST-tetrazolium was quantified using the microplate reader FLUOstar Omega.

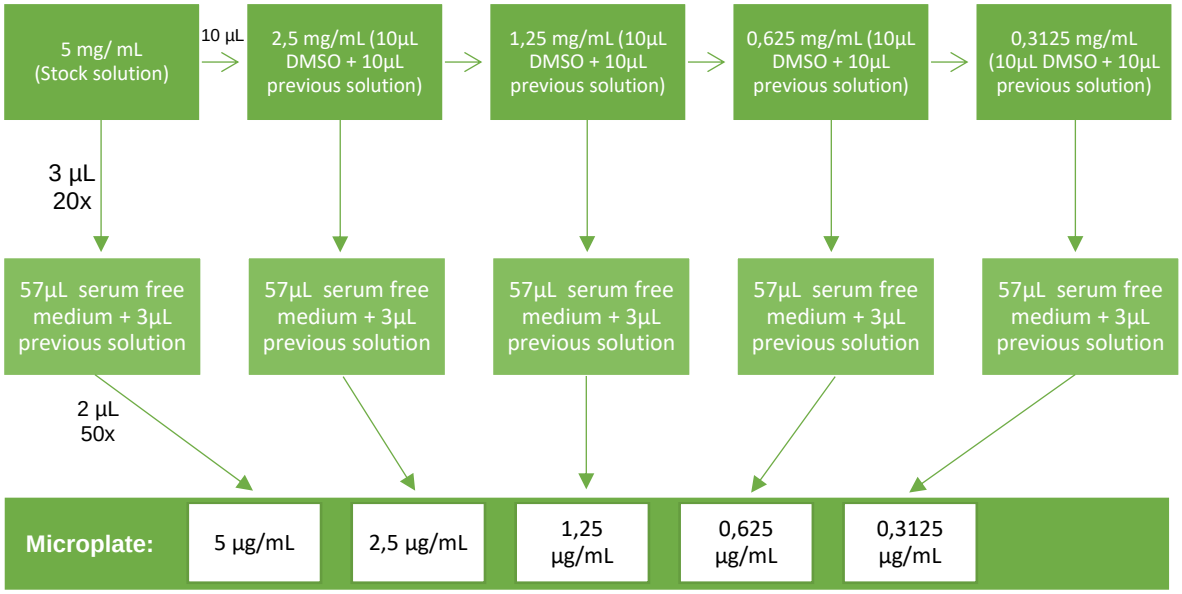

Figure 3.1: Scheme of the dilution of naphthoquinones

The collected data was analysed with the Graphpad Prism program to calculate the IC_{50} (Inhibitory concentration at half maximum). IC_{50} values are commonly used to evaluate drug potency and refers to the concentration of a drug that inhibits 50% of the activity between baseline and maximum after a specific exposure time (40).

3.4 - Antimicrobial activity of naphthoquinones against selected microorganisms

Sensibility to naphthoquinones was tested using *Micrococcus luteus* CCM 732 (Gram positive bacteria), *Escherichia coli* CCM 7929 (Gram negative bacteria) and *Saccharomyces cerevisiae* CCM 8191 (yeast).

For the culture of Gram-positive and Gram-negative bacteria the Nutrient Broth with 1% Peptone medium was used. Nutrient Broth with 1% Peptone is used as a general purpose and sterility testing media. It is composed by peptic digest of animal tissue - 10.000 g/L; beef extract - 10.000 g/L and sodium chloride - 5.000 g/L, showing a final pH of 7.4±0.2 at 25°C. Its formula was adjusted and standardized to suit performance parameters: 25 grams were suspended in 1000 ml distilled water. It was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Nutrient Broth with 1% Peptone has almost double concentration of the nitrogen sources than that used in Nutrient Broth, making it more nutritive. Nutrient Broth with 1% Peptone can be used as a sterility testing medium for aerobes against Nutrient Broth recommended for microbial limit tests as per standard pharmacopoeia. Nutrient Broth w/ 1% Peptone is a nutritionally rich medium that facilitates the growth of very low inoculum, when with fastidious microorganisms (41).

For the yeast culture, the Malt Extract Broth Base medium was used. Malt Extract Broth Base is recommended for the detection, isolation and enumeration of yeasts and moulds. It contains 17.000 g/L of malt extract and 3.000 g/L of mycological peptone, with a final pH of 5.4±0.2 at 25ºC. The formula was adjusted and standardized to suit performance parameters: 20 grams were suspended in 1000 ml distilled water and soaked for 15 minutes. It was sterilized by autoclaving at 10 lbs pressure (115°C) for 10 minutes and well mixed before dispensing. The use of malt and malt extracts for the propagation of yeasts and moulds is quite common. Malt extract provides an acidic environment and nutrients favourable for growth and metabolism of yeasts and moulds. Malt Extract Broth Base has been widely used in the maintenance, isolation and identification of fungi and it is also proposed in several pharmacopoeias as a medium for the control of sterility in pharmaceutical products, though it is mostly used for comparative morphological studies (42).

All cultures were done in 96-well microplate for determination of MIC. Concentrations of naphthoquinones varied from 1280 μg/mL to 0,025 μg/mL. Intermediate concentrations were: 640, 320, 160, 80, 40, 20, 10, 5, 4, 2, 1 and 0,5 μg/mL.

MICs were determined in vitro by the broth microdilution method according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST), using 96-well micro plates. Samples were diluted and inoculated with bacterial suspension (McFarland 0.5), and the final density was 5×10^5 CFU/ml. Microplates were incubated at air: 37±1ºC, 24±2h (*E. coli*), 37±1ºC, 48±2h (*M. luteus*) and 25±1ºC, 48±2h (*S. cerevisiae)* and bacterial growth was measured as turbidity by the microplate reader FLUOstar Omega (BMG Reader Labtech, Germany) at 600 nm. MICs were expressed as the lowest concentrations that inhibited the growth of the test bacteria compared with that of the agent-free growth control, considering inhibition as 80% of reduction of growth.

4. Results

4.1 - Cytotoxic effect on eukaryotic plant cells

Initially we tested the compounds for their cytotoxic activity against eukaryotic plant cells (from Daucus carota). The effect of naphthoquinones in the growth of eukaryotic Daucus carota cells is represented in the Figure 4.1. As can be seen, except for naphthoquinone 563, all compounds are cytotoxic in high concentration. Therefore, we decided to choose concentrations <5 µg/mL for the following experiments, once the reduction of the growth of cells is lower for almost all the molecules.

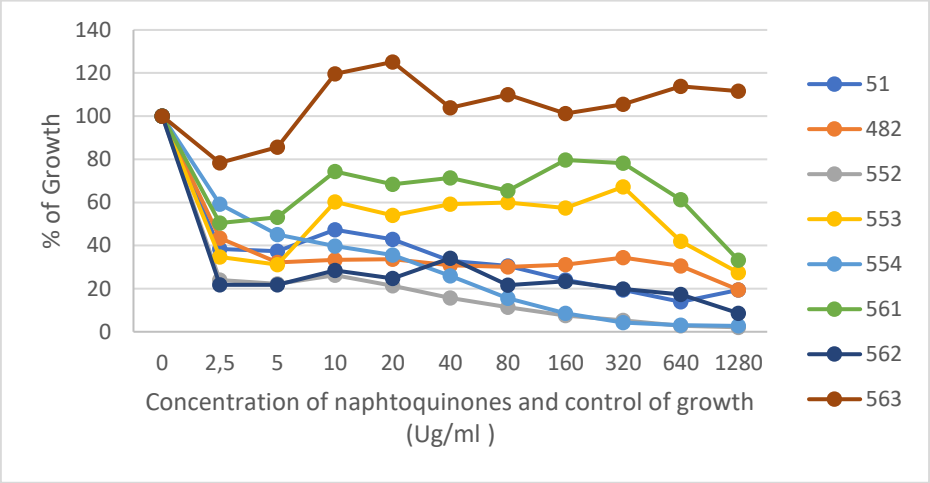

Figure 4.1: Viability of Daucus carota with each Naphthoquinone

4.2 - Cytotoxic effect on eukaryotic human cells

We used human monocytic leukaemia cell line, THP-1. For all the molecules, the following concentrations were tested: 5; 2,5; 1,25; 0,625; 0,3125 and 0 µg/mL. For molecules 51, 552 and 553 lower concentrations had to be tested additionally: 0,5; 0,25; 0,125 and 0,0625 µg/mL.

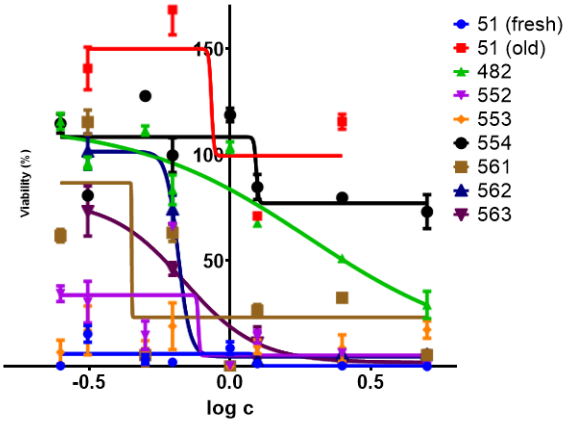

Figure 4.2: Cytotoxicity of the eight Naphthoquinones

All data was processed by Graphpad Prism software and the effect of the naphthoquinones on the viability of human cells can be observed in the Figure 4.2.

The IC_{50} calculated with Graphpad Prism is shown in Table 4.1. As so, the results show that the least toxic molecules for human cells are 554 and 482 and that the molecules 51 and 553 are the most toxic. In order to obtain statistic meaning for the calculation of IC_{50}, tests should be repeated.

Table 4.1: IC50 of the Naphthoquinones

51 (fresh)	IC_{50} < 0,25 µg/mL
482	IC_{50} = 1,842 µg/mL
552	IC_{50} ~ 0.771 µg/mL
553	IC_{50} < 0,25 µg/mL
554	IC_{50} > 5 µg/mL
561	IC_{50} ~ 0.447 µg/mL
562	IC_{50} ~ 0,654 µg/mL
563	IC_{50} ~ 0,687 µg/mL

4.3 - Antimicrobial activity of naphthoquinones against selected microorganisms

The effect of the naphthoquinones in the growth of *Escherichia coli* can be observed in the Figure 4.3 It can be seen that, for E. coli, the naphthoquinones 552, 553, 561 and 563 never reach the MIC and the relation between the concentration of Naphthoquinone and the growth of the bacteria was not established for those molecules.

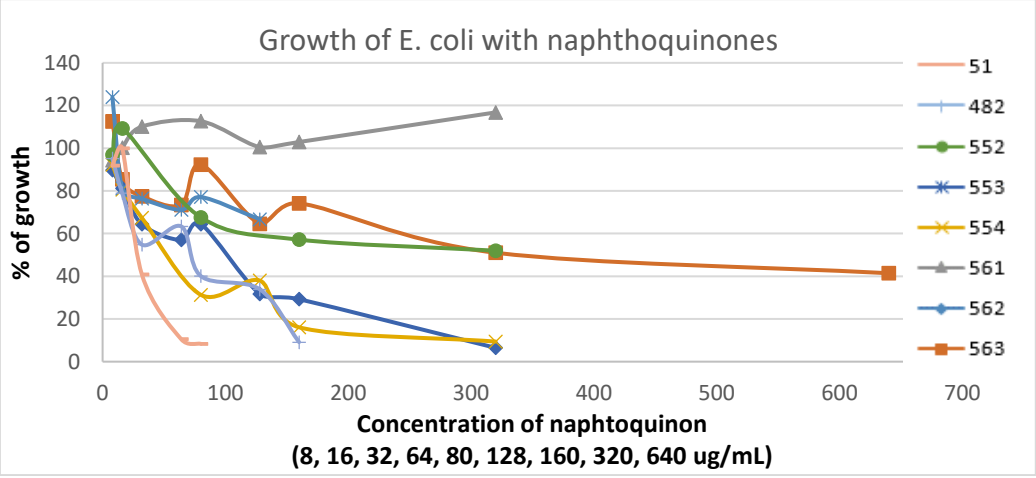

Figure 4.3: Antimicrobial activity of the Naphthoquinones against Escherichia coli

On Table 4.2 we can observe the value of MIC of the other naphthoquinones for E. coli. In order for these results to be statistically significative, tests should be repeated.

Table 4.2: MIC for each Naphthoquinone with E. coli

Molecule	MIC	
	Concentration of Naphthoquinones	% of Growth
51	64 µg/mL	10,54 %
482	160 µg/mL	9,15 %
552	-	-
553	-	-
554	160 µg/mL	16,10 %
561	-	-
562	320 µg/mL	6,49 %
563	-	-

In the Figure 4.4 it is represented the growth of *Micrococcus luteus* with the naphthoquinones. Every naphthoquinone tested was able to stop the growth of M. luteus and the values of MIC can be observed in the Table 4.3.

In order for these results to be statistically significative, tests should be repeated.

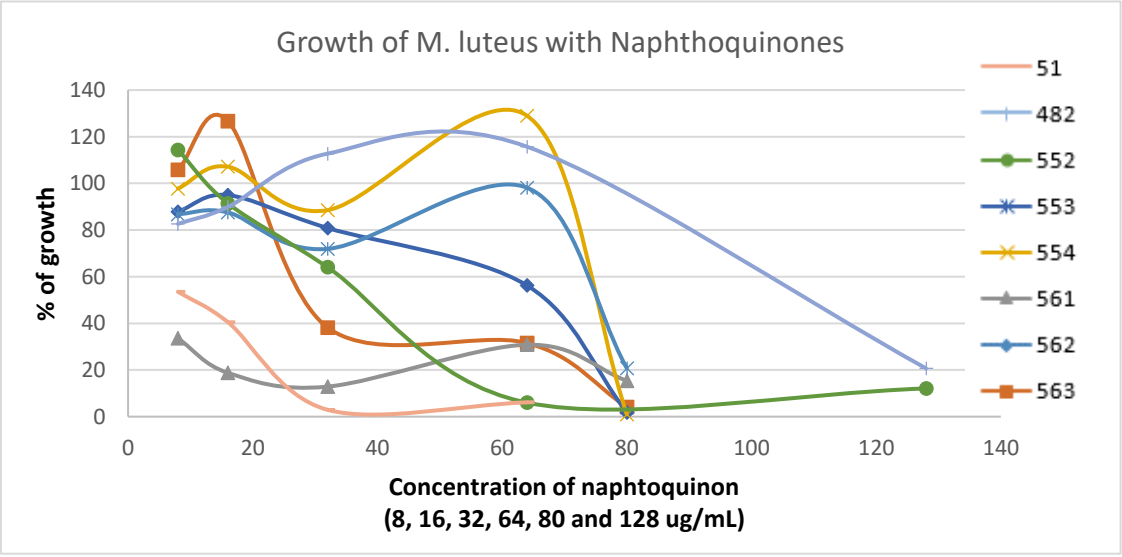

Figure 4.4: Antimicrobial activity of the Naphthoquinones against Micrococcus luteus

As can be seen in the Table 4.3 the most effective molecule is naphthoquinone 51, being able to inhibit the growth of M. luteus by 97% with a concentration of 32 µg/mL.

Table 4.3: MIC for each Naphthoquinone with M. luteus

Molecule	MIC	
	Concentration of Naphthoquinones	% of Growth
51	32 µg/mL	2,93 %
482	128 µg/mL	20,69 %
552	128 µg/mL	12,10 %
553	80 µg/mL	20,77 %
554	80 µg/mL	0,99 %
561	80 µg/mL	15,38 %
562	80 µg/mL	1,83 %
563	80 µg/mL	4,18 %

The Figures 4.5 and 4.6 represent the growth of *Saccharomyces cerevisiae* with Naphthoquinones 51, 482, 553, 561 and 563 (Figure 4.5 – lower concentrations of MIC) and with Naphthoquinones 552, 554 and 562 (Figure 4.6 – higher concentrations of MIC). As can be observed, with this yeast, the value of MIC for each Naphthoquinone is widely diverse, varying from concentrations of 0,03125 µg/mL (Naphthoquinone 51) to concentrations of 160 µg/mL (Naphthoquinone 554).

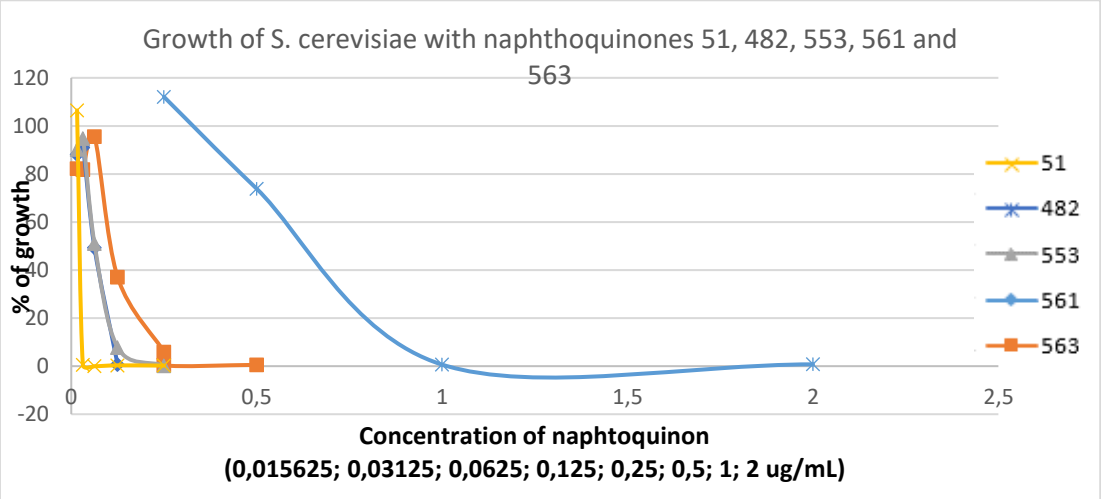

Figure 4.5: Antimicrobial activity of the Naphthoquinones 51, 482, 553, 561 and 563 against Saccharomyces cerevisiae

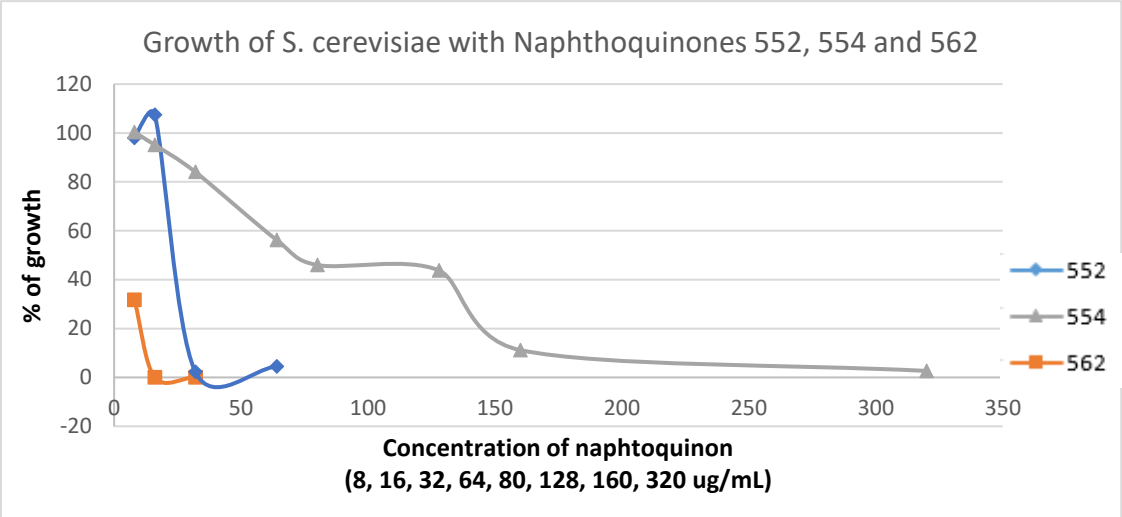

Figure 4.6: Antimicrobial activity of the Naphthoquinones 552, 554 and 562 against Saccharomyces cerevisiae

By analysing the Table 4.4, it can be seen that the most toxic naphthoquinones for S. cerevisiae is the 51 and all of the naphthoquinones, except 554, are effective in restraining the growth with this yeast with low concentrations.

Table 4.4: MIC for each Naphthoquinone with S. cerevisiae

Molecule	MIC	
	Concentration of Naphthoquinones	% of Growth
51	0,03125 µg/mL	0,59 %
482	1 µg/mL	0,65 %
552	32 µg/mL	2,25 %
553	0,125 µg/mL	7,77 %
554	160 µg/mL	11,18 %
561	0,125 µg/mL	0,66 %
562	16 µg/mL	0,06 %
563	0,25 µg/mL	5,84 %

In order for these results to be statistically significative, tests should be repeated.

5. Discussion

Every experiment of this project was only repeated once (n=1). The discussion and the conclusions presented were elaborated based on the values reached on these single experiments. To achieve statistically significant conclusions experiments should be repeated at least three times (n=3) and consequently, falling out on the time limits available to perform the experimental procedures in the lab that hosted the ERASMUS+ scholarship. Therefore, this work was focused on optimizing the approaches and selecting the best conditions for the execution of these experiments that, once repeated, will lead to the achievement of the proposed goals.

To select the range of activity of naphthoquinones were carried out viability tests with *Daucus carota*'s cells. Concentrations bellow 5 μg/mL were selected to be used on the following experiments, once the reduction of the growth of cells was lower at these concentrations for almost all the molecules.

We tested the toxicity to human cell using the THP-1 cell line which is a spontaneously immortalized monocyte-like cell line, obtained from the peripheral blood of a childhood case of acute monocytic leukaemia (43). Every compound tested shows toxicity against human cells. When IC_{50} is lower than 2 μg/ml the compound is cytotoxic (44). IC_{50} between 2 μg/ml and 89 μg/ml makes the compound is moderately cytotoxic (44). All the naphthoquinones tested are cytotoxic, except for naphthoquinone 554, that is moderately cytotoxic. Naphthoquinone 482 is really close to the threshold of moderately cytotoxicity as well.

Naphthoquinones 51, 552 and 553 required tests to be repeated, with lower concentrations: 0,5; 0,25; 0,125 and 0,0625 μg/mL, which means that those molecules were more toxic for human cells than the others. Unfortunately, these results might limit the usability of these compounds to human usage. However, their usage as antimicrobial agents to be used in plants or animal products, replacing the conventional antibiotics and possibly impacting on the mechanisms of acquired resistance, is still a possibility.

In antimicrobial tests, only four naphthoquinones, of the eight tested, were able to reduce the growth of E. coli: Naphthoquinones 51, 482, 554 and 562. Of those, Naphthoquinone 51 was the more efficient, but it also is one of the most cytotoxic for human cells. Naphthoquinone 562 needed a significant concentration to decrease the growth of the gram-negative bacteria. The naphthoquinones 482 and 554 needed intermediate concentration to do the same. They also exhibit the smaller toxicity on human cells, consequently they make good candidates to proceed for further studies.

Gram negative bacteria are the more difficult to deal with in health care, often related to nosocomial infections (45). So, it is urgent to find alternatives to address the problem of antimicrobial resistance, specifically on gram negative bacteria that are becoming resistant to all the current options for treatment, as E. *coli* and other *Enterobacteriaceae* carbapenem-resistant, also known as extended-spectrum beta-lactamases (ESBL) - producing (8). ESBL have the capacity to hydrolyse a large variety of beta-lactam antibiotics, including carbapenems (46). In fact, carbapenems have been used to effectively treat infections triggered by Enterobacteriaceae, including those ESBL-producing, until not so long ago (47). This is a serious threat because carbapenems are the last resource to threat resistant *Enterobacteriaceae* infections (48).

Naphthoquinones 482 and 554 can be further studied to prove their activity against other gram-negative bacteria, including multi-resistant strains, and may, as well, be selected to proceed to the following phases of research.

Six of the naphthoquinones reached the MIC for M. luteus culture. Only Naphthoquinones 482 and 553 did not reduced the growth up to 80%. Even so, they were on the borderline to reach it, with a decrease of 79% on the growth of this gram-positive bacteria. The most efficient molecule to reduce the growth of M. luteus was naphthoquinone 554, which is the least toxic on human cells. Of all the naphthoquinones, 554 is the one that shows the bigger potential to face gram positive bacteria.

For M. luteus the incubation time needed to be increased: initially it was 24h ± 2h, but after several poor measurements, in which there was no growth of the bacteria, incubation time was extended to 48h ± 2h and results became satisfactory. Due to lack of time, only some of the substances were tested with the right incubation time.

Naphthoquinones were highly efficient in reducing the growth of S. cerevisiae. In fact, with truly small concentrations, they were able to decrease the growth of this yeast up to 100% (Naphthoquinone 562) and 99,5% (Naphthoquinones 51, 482 and 561). Only Naphthoquinone 554 required to be used in a bigger concentration in order to reduce the growth of the yeast up to 89%. Nevertheless, as this molecule is moderately cytotoxic, the increased concentration should not be a problem.

6. Conclusions

Antimicrobial resistance is a public health problem at a global scale. We are running out of alternatives to fight bacteria, mainly gram-negative bacteria resistant to multiple antibiotics.

In order to address this problem, there are several measures proposed by WHO, such as: rationalize the use of antibiotics, by reducing the use in agriculture and livestock, better targeting the right antibiotic for the right bacteria, using small range antibiotics and reducing the use of antibiotics; invest in R&D to come up with new molecules capable of facing multi-resistant strains of specific bacteria.

After testing the new compounds, naphthoquinones, for their antibacterial activity, we can conclude that the more promising molecules, among the eight tested, are naphthoquinones 482 and 554, since they are among the naphthoquinones with better antibacterial activity and show the least toxicity on human cells. Of all the naphthoquinones tested, 554 is the one that shows a bigger potential to face the antimicrobial resistance problem. It showed activity against either gram-positive bacteria, gram-negative bacteria and yeast, having a broad range of activity. Nevertheless, naphthoquinone 482 seems to be better for fighting yeast, once it is needed in smaller concentrations to reach the MIC. Furthermore, it remains open the possibility that naphthoquinones may be used in combined antibiotic therapy for resistant bacterial strains.

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